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AN EFFECT OF 2,4-D HERBICIDE ON THE CELL SURFACE MEMBRANE OF ONION STEM EXPLANTS

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Technical Paper

AN EFFECT OF 2,4-D HERBICIDE ON THE CELL SURFACE MEMBRANE OF ONION STEM EXPLANTS

TO: J. F. McLaughlin, Director
Joint Highway Research Project

July 7, 1971

File: 9-5-3

FROM: H. L. Michael, Associate Director
Joint Highway Research Project

Project: C-36-48C

The attached Technical Paper is submitted as a report from the HPR-1 (8) research project "Research in Roadside Development", Part III, Chemical Weed Control. The paper "An Effect of 2,4-D Herbicide on the Cell Surface Membrane of Onion Stem Explants" has been authored by W. J. VanDerWoude, C. A. Lembi and D. J. Morre and is proposed for publication.

The authors plan to submit the paper to Biochemical and Biophysical Research Communications for publication. The paper is concerned with that phase of the research directed at a better understanding of the "herbicide receptor" molecules within plants. The objective of such understanding is improvement in the safe use of herbicides.

The paper is presented to the Board for approval of such publication.

Respectfully submitted,

Harold L. Michael

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Associate Director

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Summary

The activity of a plasma membrane-associated glucan synthetase of onion stem was increased by addition of the auxin herbicide 2,4-D. The effect was expressed either by pre-incubating the tissue with 2,4-D or by adding the 2,4-D to the synthetase assay. 2,4-D in the synthetase assay had little or no effect with fractions from tissue grown in the presence of 2,4-D. In the *in vitro* assay, both the synthetase and the 2,4-D required magnesium ions for activity. The 2,4-D effect was most pronounced on the incorporation of radioactivity into polysaccharides soluble in hot water; these polysaccharides representing about 20% of the total polysaccharide synthesized *in vitro*.

Lembi et al. (1971) found that plasma membrane-rich fractions isolated from maize coleoptiles by low shear homogenization and differential and sucrose gradient centrifugation, specifically bind N-1-napthylpthalamic acid (NPA), a weak auxin and inhibitor of auxin transport. In this paper, we report a response of a plasma membrane-associated enzyme of plants, glucan synthetase, to the auxin herbicide 2,4-D. The results provide further indications of specific interactions between auxins and constituents of the plant cell surface.

Methods and Materials

Plasma membrane fractions were isolated from onion (*Allium sepa*) stem according to the procedure of Lembi et al. (1971). Approximately 15 g of onion stem explants (Morré, 1970) were homogenized in 35 ml of a freshly prepared medium consisting of 0.5 M sucrose, 0.1 M K_2HPO_4, 1 mM dithiothreitol and 0.02 M EDTA in coconut milk as the solvent and adjusted to pH 6.5 with NaOH. Nuclei and other endogenous cellular components of the coconut milk were removed by high speed centrifugation (100,000 g, 90 min) before preparing the medium. Homogenates were prepared in the cold using a mechanized razor blade chopper (Morré, 1971). Cell walls and unbroken cells were removed by filtration through a single layer of miracloth (Chicopee Mills, New York), and the filtered homogenate was centrifuged for 30 min at 10,000 g (Spinco L2-65B, SW 50.1 rotor) to remove nuclei, plastids, mitochondria and large membrane fragments. The supernatant containing microsomes and the majority of the cytoplasmic membrane fragments was then sedimented at 44,000 g onto a two-layered cushion consisting of

1.5 M and 2.0 M sucrose in coconut milk to which 1 mM dithiothreitol had been added. The supernatant was removed and replaced by a step gradient of 0.5, 0.8, 1.1, 1.2 and 1.3 M sucrose in coconut milk + 1 mM dithiothreitol. The gradient was then centrifuged for 90 min at 100,000 g. Plasma membranes were recovered from the 1.1/1.2 M interface of the gradient, resuspended in homogenization medium and pelleted for 20-30 min at 100,000 g. The plasma membrane fragments were identified by electron microscopy after differential staining with a mixture of phosphotungstic acid and chromic acid which specifically and characteristically stains the plant cell membrane (Morré et al., 1970; Lembi et al., 1971, in preparation).

The glucan synthetase assay is adapted from that of Ordin and Hall (1967) and is based on the incorporation of isotopically labeled sugars from appropriate sugar nucleotides into polysaccharides of varying solubility. The substrate concentration was increased to saturate the substrate requirements for the synthetases involved with glucose incorporation into lipid-soluble compounds and intermediates (Pinsky and Ordin, 1969). This activity of onion stem is characterized by a high affinity for UDP-glucose (Km = 2.5 X 10^{-8} M) and competes with the glucan synthetases at low substrate concentrations (Lembi et al., in preparation). As shown previously by Ordin and Hall (1968) and Feingold et al. (1958) predominantly beta 1,3 linkages are produced from UDP-glucose at these high substrate concentrations.

Fractions for assay were resuspended in 0.1 M tris-HCl containing 4 mM disodium EDTA and 1 mM dithiothreitol, final pH 8.0, at a concentration of 1-4 mg protein/ml. The assay medium contained 0.3 mμ moles UDP-glucose-C^{14} (227 μC/μmole), 200 mμmoles unlabeled UDP-glucose, 10 μmoles Tris-HCl,

pH 8.0, 0.4 μmoles disodium EDTA, 0.1 μmoles dithiothreitol, 4 μmoles $MgCl_2$, 2 μmoles cellobiose (as the acceptor) and an aliquot of the fraction to provide 50-200 μg protein in a final volume of 200 μl. Incubations were for 15 min at 25°.

Prior to fractionation of the polysaccharide synthesized into hot water soluble-, lipid soluble-, sodium hydroxide soluble- and sodium hydroxide insoluble-constituents, cellulose powder (Sigmacell, Sigma Chemical Company, St. Louis, Missouri), 1-2 mg, was added to each assay tube. The mixture was then extracted 3 times, 5 min each, with 1 ml aliquots of water at 95-100°. Insoluble polysaccharides were recovered after each extraction by centrifugation at 25,000 g for 12 min. Hot water insoluble polysaccharides were recovered from the hot water extracts by addition of 10 mg ficol (mol wt. 400,000) followed by precipitation from cold 70% ethanol. The hot water insoluble residue was extracted once with 1 ml of methanol-chloroform (2:1, v/v) and once with 1 ml of absolute methanol. The methanol-chloroform and methanol extracts were combined to provide a measure of the synthesis of lipid-soluble sugar derivatives and intermediates (Pinsky and Ordin, 1969). After removal of lipids, the residue was then extracted twice with 0.25 ml aliquots of hot 1 N sodium hydroxide for 5 min followed by 0.75 ml of cold water. The combined alkali extracts (including the water wash) were evaporated to dryness for direct determination of radioactivity on planchets. The alkali insoluble residue is the "cellulose" fraction. Self absorption corrections were determined and applied to each of the fractions.

Results

The specific activity of a marker enzyme UDP-glucose: glucose glucosyltransferase (glucan synthetase), concentrated in plasma membrane fractions isolated from onion stem explants (Lembi et al., in preparation), is markedly increased by incubating the explants in the presence of the synthetic plant growth regulator 2,4-D (Table I). With tissue incubated for 12-16 hours in the presence or absence of 5 μM 2,4-D, the specific activity of the glucan synthetase of plasma membranes isolated from the 2,4-D-incubated tissue us increased approximately 25% over that of plasma membranes from control tissues incubated in the absence of 2,4-D.

To demonstrate that a similar response results from the in vitro addition of the auxin, plasma membranes were isolated from tissues incubated in the presence or absence of 5 μM 2,4-D. Each of the preparations was then assayed in the presence or absence of 5 μM 2,4-D added to the enzyme assay medium (Table II). A consistent stimulation of 7 to 15% was observed in the synthesis of hot water insoluble glucans when 2,4-D was added to plasma membranes isolated from control tissues. With plasma membranes isolated from 2,4-D incubated tissues, incubation with 2,4-D had little or no addition effect on the glucan synthetase activity (Table II).

The products of the synthetase assay are heterogeneous and not all fractions are affected similarly by the presence of 2,4-D in the enzyme assay. After fractionation, the largest 2,4-D response of the hot water insoluble fraction is expressed in the fraction insoluble in HOT NaOH; the fraction

soluble in hot NaOH showing much less of an effect (Table III). There was no effect of 2,4-D on glucose incorporation into the lipid soluble fraction. The glucan fraction soluble in hot water, a fraction not included in data of Tables I and II, showed the greatest stimulation (30%) by 2,4-D added to the glucan synthetase assay.

The plasma membrane-associated glucan synthetase was markedly stimulated by magnesium ions (Table IV) and the 2,4-D-induced stimulation of activity was not observed in the absence of magnesium ions. In the absence of magnesium ions, 2,4-D was without effect or inhibited the activity of the glucan synthetase. Again, the greatest stimulation was shown by the hot water soluble fraction rather than the hot water insoluble fraction (30% vs. 11% stimulation by 2,4-D).

A comparison of the relative effectiveness of <u>in vitro</u> and <u>in vivo</u> incubations with 2,4-D on the production of the hot water soluble glucan by isolated plasma membranes is given in Table V. In 3 experiments, a stimulation of 30 to 65% resulted from the inclusion of 2,4-D in the glucan synthetase assay with plasma membranes isolated from tissue incubated in the absence of 2,4-D. The inclusion of 2,4-D in the glucan synthetase assay of plasma membranes isolated from tissue incubated in the presence of 2,4-D had no additional effect except in Expt. II of Table V where incubation of the tissue with 2,4-D was not effective (-15% stimulation). In this experiment, addition of 2,4-D to the synthetase assay resulted in an apparent stimulation of 80% in the synthesis of hot water soluble glucans.

Discussion

A 50% enhancement of glucan synthetase activities by auxin pretreatment was previously reported for pea epicotyls (Abdul-Baki and Ray, 1971); an activity localized within the Golgi apparatus of this tissue by Ray et al. (1969). A similar enhancement, although not as great, appears to be associated with the plasma membrane-associated glucan synthetase of onion stem. Additionally, we show that 2,4-D added to the isolated plasma membrane fragments increases the specific activity of the glucan synthetase when the plasma membrane fragments are isolated from tissues which have not been pre-treated with auxin. Except for one experiment (Expt. II of Table V), 2,4-D added to plasma membranes isolated from tissues incubated with 2,4-D resulted in no additional stimulation. Although we do not rule out an effect of 2,4-D on the synthesis of glucan synthetase *in vivo*, at least a significant portion of the stimulation appears to result from a direct effect on the plasma membrane-associated synthetase.

The *in vitro* response to 2,4-D is most marked with the hot water soluble polysaccharide fraction, a fraction specifically enhanced by auxin treatment of growing tissues (Bonner, 1961; Ray, 1962; Morré and Olson, 1965). Here, the response to 2,4-D added *in vitro* to the glucan synthetase assay was equivalent to results obtained with plasma membranes isolated from tissues incubated 12 to 16 hours in the presence of 2,4-D. Although related to an artificial system of wall fractionation (Albersheim, 1965), the results are important in that a response to auxin has been obtained by adding the auxin to an *in vitro* system.

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TABLE I

IN VIVO STIMULATION OF PLASMA MEMBRANE-ASSOCIATED GLUCAN SYNTHETASE BY 2,4-D PRETREATMENT OF ONION STEM TISSUE

Onion stem explants (Morré, 1970) were incubated for 12-16 hours at $25^{\circ} \pm 5\mu M$ 2,4-D. Plasma membrane fractions were isolated by the method of Lembi et al. (1971). Units of specific activity of the isolated fractions are m moles glucose incorporated into total hot water-insoluble polysaccharide/hr/mg protein.

Tissue preincubation	Specific activity			
	Expt. I	Expt. II	Expt. III	Expt. IV
None	319	321	384	324
$5\mu M$ 2,4-D	410	391	458	410
Δ 2,4-D %	29	22	19	26

IN VITRO 2,4-D STIMULATION OF PLASMA MEMBRANE-ASSOCIATED GLUCAN SYNTHETASE COMPARING ONION STEM TISSUE PRETREATED ± 2,4-D

Plasma membrane fractions were isolated from stem explants and units of specific activity are as described in Table 1. 2 μM 2,4-D was then added to the enzyme assay to test for *in vitro* stimulation of the membrane-associated synthetase.

Auxin added			Specific activity					
Tissue incubation	Synthetase assay		Expt. II	Expt. III	Expt. IV	Expt. V	Expt. VI	Ave.
None	None		321	384	324	342	377	350
	5 μM 2,4-D		344	443	373	366	414	388
	Δ 2,4-D, %		+7	+15	+15	+7	+10	+11
5 μM 2,4-D	None		391	458	410	---	---	420
	5 μM 2,4-D		401	483	393	---	---	426
	Δ 2,4-D, %		+3	+5	-4	--	--	+1

TABLE III

SUBFRACTIONATION OF POLYSACCHARIDES OF THE IN VITRO ASSAY AND EFFECT OF 2,4-D ON INCORPORATION OF RADIOACTIVITY INTO VARIOUS SUBFRACTIONS

Onion stem explants were incubated 12-16 hours at 25° in the absence of 2,4-D. Plasma membrane fractions were isolated and glucan synthetase activity was assayed ± 5·μM 2,4-D added directly to the enzyme assay. Details of the polysaccharide fractionation are given in the text.

		mμmoles glucose incorporated/hr/mg protein			
Auxin added to		Fractions soluble in			Hot NaOH
synthetase assay	Expt.	Hot water	$CHCl_3$-MeOH	Hot NaOH	Insoluble
None	III	85	21	104	280
	V	86	22	138	214
	VI	37	14	147	230
	Ave.	70	20	130	240
5μM 2,4-D	III	123	19	126	317
	V	96	21	133	233
	VI	50	19	143	271
	Ave.	90	20	135	274
Δ 2,4-D, %		+30	0	+4	+14

TABLE IV

EFFECT OF MAGNESIUM IONS ON PLASMA MEMBRANE-BOUND GLUCAN SYNTHETASE AND THE RESPONSE TO 2,4-D

Plasma membrane fractions were isolated from stem explants and units of specific activity are as described in Table III. Average of 2 experiments.

Auxin added to synthetase assay	Synthetase assay medium	mμmoles glucose incorporated/hr/mg protein	
		Hot water soluble	Hot water insoluble
None	$-Mg^{++}$	61	80
	Complete	85	363
5μM 2,4-D	$-Mg^{++}$	56	81
	Complete	110	404

TABLE V

COMPARISON OF IN VITRO AND IN VIVO STIMULATION BY 2,4-D OF THE SYNTHESIS OF HOT WATER SOLUBLE POLYSACCHARIDES BY THE PLASMA MEMBRANE-ASSOCIATED GLUCAN SYNTHETASE

Plasma membrane fractions were isolated from stem explants incubated as described in Table 1. The unit of specific activity is mμmoles glucose incorporated into total hot water soluble polysaccharide/hr/mg protein.

Auxin added					
Tissue incubation	Synthetase assay	Specific activity			
		Expt. II	Expt. III	Expt. IV	Ave.
None	None	95	83	103	90
	5μM 2,4-D	120	111	169	134
		Δ 2,4-D, % +45	+64	+34	+49
5μM 2,4-D	None	72	115	190	126
	5μM 2,4-D	129	112	185	142
		Δ 2,4-D, % +80	-3	-3	+12
Δ Tissue incubation with 2,4-D, %		-15	+39	+85	+40